Biotechnology Letters **25:** 301–305, 2003.

Improved substrate specificity of water-soluble pyrroloquinoline quinone glucose dehydrogenase by a peptide ligand

Hiromi Yoshida, Yukiko Yagi, Kazunori Ikebukuro & Koji Sode*
Department of Biotechnology, Faculty of Technology, Tokyo University of Agriculture and Technology, 2-24-16 Nakamachi, Koganei, Tokyo 184-8588, Japan
**Author for correspondence (Fax: +81-42-388-7027; E-mail: sode@cc.tuat.ac.jp)*

Received 3 October 2002; Revisions requested 28 October 2002; Revisions received 6 December 2002; Accepted 9 December 2002

Key words: peptide ligand, phage display, PQQ-glucose dehydrogenase, quaternary structure design, substrate specificity

Abstract

A new approach in altering the substrate specificity of enzyme is proposed using glucose dehydrogenase, with pyrroroquinoine quinone (PQQGDH) as co-factor, as the model. This approach is based on the selection of random peptide phage displayed library. Using an M13 phage-display random peptide library, we have selected peptide ligands. Among the peptide ligands, a 7-mer peptide, composed of Thr-Thr-Ala-Thr-Glu-Tyr-Ser, caused PQQGDH substrate specificity to decrease significantly toward disaccharides, such as maltose and lactose, while a smaller effect was observed toward glucose. Consequently, this peptide narrowed the substrate specificity of PQQGDH, without a significant loss of the enzyme activity.

Introduction

Glucose dehydrogenases (GDHs) possessing pyrroloquinoline quinone (PQQ) as the prosthetic group have been highly focused as an ideal enzyme for mediator-type, glucose sensor constituent because of its inherent characteristics and independence of O_2 (Kost *et al.* 1998, Tang *et al.* 2001). However the improvement of thermal stability and substrate specificity are further required for PQQGDHs, comparing the characteristic properties of conventionally utilized glucose sensing enzyme, glucose oxidase.

We have been studying protein engineering of PQQGDH to improve their enzymatic properties as an enzyme sensor constituent. We reported the construction of a mutant PQQGDH with increased thermal stability by error prone PCR (Sode *et al.* 2000) and fabrication of a glucose sensor using this mutant (Takahashi *et al.* 2000). We have reported that the alteration of enzymatic properties of PQQGDH is possible by protein engineering, such as site directed mutagenesis (Igarashi *et al.* 1999, Sode *et al.* 2000) or construction of a chimeric enzyme (Yoshida *et al.* 2000). These involve novel designs of the enzyme focusing on its native tertiary and quaternary structures. Besides, in nature, several enzymes are composed of subunits to form active quaternary structures, thereby showing a higher order of the function, e.g. electron transfer, protein folding like chaperon, ion channeling etc. We propose a novel strategy of protein engineering, termed quaternary structure engineering, by which artificial subunits are developed to control or even create new function of the target protein. However, the design of an adequate interface for protein, protein interaction, which are not forming a complex, is still a remote possibility. Here, we propose a new concept to construct an artificial 'subunit' which can control the substrate specificity of enzyme. There is no report on alteration of enzymatic properties of PQQGDH by addition of biomolecules, proteins, nucleic acids and peptides as ligands which then bind to the enzyme.

Phage display is well known as a powerful method for screening of peptide ligands to various target proteins (Smith 1985). There have been many reports concerning the identification of peptide hormone (Low-

man *et al.* 1991), protease substrates or inhibitors by isolation of new peptide ligands (Houimel *et al.* 1999). Moreover, the screening of peptide ligands which bind to the enzyme and increase its activity (Wu *et al.* 2000) or its thermostability (Chakravarty *et al.* 2000) have been reported. Utilizing the phage display method, we have screened random peptide ligands which bind to PQQGDH and can alter its enzymatic properties. In this paper, we report the alteration of the substrate specificity of PQQGDH in the presence of the peptide ligand selected from M13 phage peptide library and this is one of the approaches for quaternary structure design of enzyme.

Materials and methods

Phage selection

Ph.D.-7 Phage Display Peptide Library Kit and Ph.D.-12 Phage Display Peptide Library Kit (New England Biolabs, Beverly, MA) were used for biopanning. The coating solution containing 200 μg purified water-soluble PQQ glucose dehydrogenase (Igarashi *et al.* 1999) in 0.1 M $NaHCO_3$ buffer (pH 8.6) was put into a petri dish (85 mm diam.) and was incubated overnight at 4 °C. After removal of the coating solution, the dish was filled with the blocking buffer [0.1 M $NaHCO_3$ (pH 8.6), 5 mg BSA ml^{-1}, 0.02% NaN_3] for 1 h at 4 °C. The dish was washed 6 times with TBST [50 mM Tris/HCl (pH 8) containing 150 mM NaCl and 0.1% (v/v) Tween20] and subsequently coated with the phage solution containing 2×10^{11} random phage display library in TBST and incubated for 60 min at room temperature. The non-bound phages were removed by washing with TBST 10 times and the bound phage was eluted with 0.1 M glycine/HCl (pH 2.2) containing 1 mg BSA ml^{-1}. Eluted phage solutions were neutralized with 1 M Tris/HCl (pH 9.1) and amplified in *Escherichia coli* ER2738 for the next biopanning. The above panning process was repeated six times for Ph.D.-7 Phage Display Peptide Library and 5 times for Ph.D.-12 Phage Display Peptide Library. From third round of biopanning with Ph.D.-7 Phage Display Peptide Library, 15 phages were randomly selected, and 10 phages in each round from 4th to 6th round were selected for sequence analysis as bound phage clones and analyzed according to instruction manual. In case of Ph.D.-12 Phage Display Peptide Library, 10 phages were selected from 4th and 5th round and their sequence analysis was carried out.

Investigation of the effect of peptide ligands on the enzymatic activity of PQQGDH

Individual phage solution of 15 selected phage clones (No.3-1 to No.3-15) from the 3rd round was prepared as follows. Each phage clone was inoculated into cultured *E. coli* ER2738 and incubated for 4.5 h at 37 °C. The supernatant of each culture was obtained by centrifugation and used for quick GDH assay as a peptide displaying phage solution. Quick GDH assay was performed according to our previous study (Igarashi *et al.* 1999) except for the incubation in the presence of phage solution. The GDH activity in the presence of individual 15 phage solutions was measured by comparing the rate of the color change of 2,6-dichlorophenolindophenol (DCIP) from blue to clear in the presence of 50 mM glucose and 25 mM lactose.

To analyze further the effect of peptide ligand on enzymatic activity of PQQGDH, four different synthetic peptides consisting of seven amino acid residues or 12 amino acid residues were prepared by Sawady Technology Co., Ltd. (Tokyo, Japan) and those amino acid sequences are mentioned in the Results and discussion section.

For the GDH assay utilizing each synthetic peptide, 1.14×10^{-13} mol (5.7×10^{-10} M) of purified PQQGDH was incubated in 10 mM MOPS/NaOH buffer (pH 7) containing 1 mM $CaCl_2$ and 1 μM PQQ for 10 min and subsequently incubated in the presence of 2.5×10^{-10} mol (1.3×10^{-6} M) of the 7-mer peptides or 1.6×10^{-11} mol (8×10^{-8} M) of the 12-mer peptides for 10 min at room temperature. GDH activity was measured using 0.6 mM phenazine methosulfate (PMS) and 0.06 mM DCIP. Decrease in absorbance of DCIP at 600 nm, in the presence of synthetic peptide at various concentrations of each substrate, was monitored and kinetic parameters of PQQGDH were as described previously (Igarashi *et al.* 1999).

Results and discussion

Sequence analysis for screening of peptide peptide ligands interacting with PQQGDH

Amino acid sequences of the randomly selected phage clones from the 3rd round to the 6th round using Ph.D.-7 Phage Display Peptide Library are summarized in Figure 1. Finally selected 10 clones showed the unique sequence (NATLTRL) in 6th round. This

A The sequences of 7- mer peptides selected for PQQGDH

clone number	3rd round	4th round	5th round	6th round
1	SNTQLDQ	NATLTRL(3)	NATLTRL(7)	NATLTRL(10)
2	AAKTWNA	VTYRAPA(2)	SILPYPY(3)	
3	HQLTKTL	HTLNWTH		
4	TELLWT	HTPNLHP		
5	TGGSYGS	QGIAEFR		
6	LPGSPWL	QLHPLKP		
7	TLQPPLL	LGAPPTG		
8	SPNWNLT			
9	LTPNPY			
10	NDTRPPR			
11	YPLNHSP			
12	QHSSPSY			
13	TMTWTSQ			
14	TTATDYS			
15	SAPKTTF			

B The sequences of 12- mer peptides selected for PQQGDH

clone number	4th round	5th round
1	LPAPLLPAAPLY(4)	LPAPLLPAAPLY(9)
2	SISKSPWSLLSP	
3	WAPGSMPTSRLA	
4	GNYWSKQHSYHS	

Fig. 1. Amino acid sequences of 7-mer (A), and 12-mer (B), peptide ligands selected for PQQGDH in each round. The number in brackets means the number of clones showing the same amino acid sequence. The peptides bearing underlined amino acid sequences are synthesized for further analysis.

was designated as No. 4-1 since this sequence appeared first time in 4th round. At the 5th round, we also obtained frequently appearing peptide and named it as No. 5-1 (SILPYPY). In addition to these highly frequently appeared sequences, No. 3-1 (HQLTKTL) and No. 3-14 (TTATDYS) were also selected from 3rd round panning for further detailed analyses using synthetic peptides. As for the panning using Ph.D.-12 Phage Display Peptide Library, randomly selected 10 phage clones from 5th round were identical (No. 12; LPAPLLPAAPLY), and was selected for further analyses.

Thus, 4 synthetic peptides consisting of 7 amino acid residues (No. 3-3, No. 3-14, No. 4-1 and No. 5-1) and one synthetic peptide with 12 amino acid residues (No. 12) were prepared for further analyses.

Effect of synthetic peptide ligands on substrate specificity of PQQGDH

We first measured the GDH activity toward various substrates in the presence of each synthetic 7-mer peptide ligand, and investigated the impact of these peptides on the substrate specificity of PQQGDH. Table 1 shows the substrate specificity of PQQGDH, in the presence or absence of these peptides, by comparing relative activity for each substrate at 25 mM using glucose as the control. PQQGDH is characteristic in utilizing disaccharides, such as lactose as maltose as the substrate, and the target of engineering is to decrease its activity toward these disaccharides. The addition of peptide No. 4-1, resulted in the increase in the relative activity toward 3-*o*-m-D-glucose, although similar activity was observed toward lactose and maltose compared with the enzyme activity in the absence of peptides. The most significant difference was observed in the substrate specificity in the presence of No. 3-14 peptide. The presence of No. 3-14 peptide decreased the relative activity toward both maltose (87%→61%) and lactose (68%→33%). Therefore, No. 3-14 was chosen for further detailed analyses together with the peptide selected from 12-mer library, No. 12-1.

Table 1. Substrate speicificity of PQQGDH with peptides.

Substrate	Relative activity of PQQGDH (%)				
	Wild type	No. 5-1	No. 4-1	No. 3-3	No. 3-14
Glucose	100	100	100	100	100
2-Deoxy-glucose	5	2	5	3	1
Mammose	15	16	10	12	6
Allose	77	51	80	62	52
3-*o*-m-D-glucose	65	82	114	78	44
Galactose	20	13	20	8	10
Xylose	13	11	15	13	10
Lactose	68	49	68	64	33
Maltose	87	79	85	76	61

Substrate concentration: 25 mM.

Figure 2 shows the effect of the presence of the peptide ligand No. 3-14 and 12-mer peptide ligand, No. 12 on the PQQGDH activity toward glucose and maltose. As shown in Figure 2, the presence of both peptide ligands showed inhibitory effect toward GDH activity. However, the inhibitory effects toward glucose and maltose were different, and much significant toward maltose. The observed specific activity toward glucose decreased to 80% and 60% in the presence of No. 3-14 or No. 12-1 peptide, respectively, whereas the specific activity toward maltose decreased to less than 50% in the presence of both peptides. Therefore, the different impact in inhibitory effect caused by these peptides resulted in narrowing substrate specificity of PQQGDH.

The V_{max}/K_m values of PQQGDH for different substrates with or without the peptide ligand No. 3-14 and No. 12 are summarized in Table 2. Each V_{max}/K_m value to glucose was considered as 100% in the presence or absence of the peptide ligand and we evaluated the substrate specificity of PQQGDH depending on the peptide ligand by comparing each relative value

Table 2. The V_{max}/K_m values of PQQGDH to different substrates in the presence of the peptide.

	Wild type		No. 3-14		No. 12	
	V_{max}/K_m (U mg^{-1} mM^{-1})	Relative values (%)	V_{max}/K_m (U mg^{-1} mM^{-1})	Relative values (%)	V_{max}/K_m (U mg^{-1} mM^{-1})	Relative values (%)
Glucose	70	100	52	100	38	100
Lactose	31	44	12	23	31	82
Allose	32	46	17	33	21	55
Maltose	68	97	18	35	17	45
Galactose	1	1	1	2	1	3
3-o-m-D-glucose	21	30	14	28	14	36
Mammose	38	55	55	105	35	93
Xylose	4	5	2	3	2	6

Relative value is shown as percentage of V_{max}/K_m value of PQQGDH for each substrate to that for glucose.

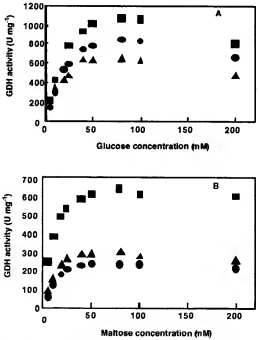

Fig. 2. GDH activity for glucose (A), and for maltose (B), in the presence of peptide ligands. GDH activity of wild type (■), with the peptide ligand No. 3-14 (●), with the peptide ligand No. 12 (▲) were plotted on different glucose concentrations.

of V_{max}/K_m for various substrates. In the presence of peptide ligand No. 12, the relative values of V_{max}/K_m for various substrates showed higher values compared with those values in the absence of peptide ligand except for the value for maltose. The relative values of V_{max}/K_m for maltose in the presence of peptide ligand No. 12 showed 45%, and that for maltose in the absence of peptide ligand was 97%. By addition of peptide ligand No. 12, the substrate specificity of PQQGDH for maltose was improved.

In case of the addition of peptide ligand No. 3-14, the relative value of V_{max}/K_m for lactose was 23%, which is approximately half of that value in the absence of peptide ligand. Moreover, the relative values of V_{max}/K_m for maltose was also low, 35% which is approximately one third of that value in the absence of peptide ligand. The enzymatic activity of PQQGDH for disaccharide was decreased in the presence of peptide ligand No. 3-14, resulting in the improvement of substrate selectivity of PQQGDH for glucose.

The selected peptide ligand No. 3-14 from the phage peptide library caused the alteration of substrate specificity of this enzyme and improved it for glucose. The presence of peptide ligand No. 3-14 suggested some interaction with PQQGDH and the possibility of inhibition to substrate interaction with PQQGDH, considering the dependence of GDH activity on peptide ligand concentration. The peptide ligand interacting with PQQGDH might show different interactions with each substrate and then might show different inhibitions to each one, which might result in significant alteration of substrate specificity of PQQGDH.

PQQGDH is composed of 478 amino acid residues and contains 11 Arg and 37 Lys as positively charged amino acid residue (10% of all amino acid residues). Among these positively charged residues, exposed residues on the enzyme surface are 7 Arg and 22 Lys. The peptide ligand No. 3-14 containing Asp as negatively charged residue and has relatively higher hydrophilicity than other selected synthetic peptide ligands, therefore the peptide ligand No. 3-14 may in-

teract with positively charged residues or hydrophilic residues forming active site of PQQGDH.

Finally selected amino acid sequences in the biopanning, No.4-1 from Ph.D.-7 Phage Display Peptide Library and No. 12 from Ph.D.-12 Phage Display Peptide Library did not show significant effect on GDH activity compared with the effect by No. 3-14. These peptide ligands containing highly hydrophobic residue might have an interaction with hydrophobic region of PQQGDH but no interaction with active site of the enzyme.

In conclusion, we have improved the substrate specificity of GDH by introducing peptide ligands. This achievement indicates that the engineering of enzymatic property can be achieved by designing new synthetic subunits of the enzyme, which can be called *quaternary structure engineering*. In this study we used 7-mer and 12-mer peptide libraries, so that the peptides of this length might not form a rigid conformation. Therefore partial and local hydrophobic and/or electrostatic interaction will dominate, resulting in weak interaction to target molecules and, consequently, significant alterations in enzyme properties should not occur. Therefore screening for longer peptide ligands should lead to further improvements in the performance of the engineered enzyme.

Acknowledgements

The authors thank Dr Atsushi Sato in RIKEN for helpful discussion. This work was supported by Grant in Aid for Scientific Research, Special Research Promotion (No. 13031021) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Chakravarty S, Mitra N, Surolia A, Varad AR (2000) Protein stabilization through phage diplay. *FEBS Lett.* **476**: 296–300.

Houimel H, Mach JL, Corthesy, Fisch I (1999) New inhibitors of *Helicobacter pylori* urease heloenzyme selected from phage-displayed peptide libraries. *Eur. J. Biochem.* **262**: 774–780.

Igarashi S, Ootera T, Yoshida H, Witarto AB, Sode K (1999) Construction and characterization of mutant water soluble (PQQ) glucose dehydrogenase with altered K_m value-site directed mutagenesis studies on the putative active site. *Biochem. Biophys. Res. Commun.* **264**: 820–824.

Kost GJ, Vu HT, Lee JH, Bourgeois P, Kiechle FL, Martin C, Miller SS, Okorodudu AO, Podczacy JJ, Webster R, Whitlow KJ (1998) Multicenter study of oxygen-insensitive handheid glucose point-of-care testing in clinical care/hospital/ambulatory patients in the United States and Canada. *Crit. Care Med.* **26**: 581–590.

Lowman HB, Bass SH, Simpson N, Wells JA (1991) Selecting high-affinity binding proteins by monovalent phage library. *Biochemistry* **30**: 10832–10838.

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the surface of the virion. *Science* **228**: 1315–1317.

Sode K, Ootera T, Shirahane M, Witarto AB, Igarashi S, Yoshida H (2000) Increasing the thermal stability of the water-soluble pyrroloquinoline quinone glucose dehydrogenase by single amino acid replacement. *Enz. Microb. Technol.* **26**: 820–824.

Takahashi Y, Igarashi S, Nakazawa Y, Tsugawa W, Sode K (2000) Construction and characterization of glucose enzyme sensor employing engineered water soluble (PQQ) glucose dehydrogenase with improved thermal stability. *Electrochemistry* **68**: 907–911.

Tang Z, Louie RF, Lee JH, Lee DM, Miller EE, Kost GJ (2001) Oxygen effects on glucose meter measurement with glucose dehydrogenase- and glucose oxidase-based test strips for point-of-care testing. *Crit. Care Med.* **29**: 1062–1070.

Wu P, Leinonen J, Koivunen E, Lankinen H, Stenman UH (2000) Identification of novel prostate-specific antigen-binding peptides modulating its enzyme activity. *Eur. J. Biochem.* **267**: 6212–6220.

Yoshida H, Iguchi T, Sode K (2000) Construction of multi-chimeric pyrroloquinoline quinone glucose dehydrogenase with improved enzymatic propertiesand application in glucose monitoring. *Biotechnol. Lett.* **22**: 1505–1510.